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Soluble reduced nicotinamide adenine dinucleotide oxidase from *Bacillus cereus* T spores and vegetative cells. I. Purification

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Spores and vegetative cells of *Bacillus cereus* T were disrupted by two procedures and soluble extracts prepared from the ruptured cells. Reduced nicotinamide adenine dinucleotide (NADH₂) oxidases were purified from the extracts by ammonium sulfate fractionation, ion exchange on hydroxylapatite, and preparative acrylamide gel electrophoresis. The electrophoresis step revealed the presence of two distinct components with NADH₂ oxidase activity in soluble extracts of spores while vegetative cell extracts contained only one. The faster moving component in spore extracts constituted about 30% of the NADH₂ oxidase activity recovered and was identical with the vegetative cell enzyme in electrophoretic mobility on acrylamide gel. The slower moving spore component accounted for 70% of recovered activity and was found in soluble extracts regardless of the procedure used to rupture spores.

Introduction

A number of enzymes capable of oxidizing reduced nicotinamide adenine dinucleotide (NADH₂) in spores and vegetative cells of *Bacillus cereus* T have been described. Vegetative cells possess a particulate cytochrome system while functional electron transport in spores proceeds mainly via a soluble oxidase (5). The soluble NADH₂ oxidase from spores has been partially purified and reportedly requires flavin mononucleotide (FMN) for activity (6). Recently, there has been considerable interest in the comparison of spore enzymes with their vegetative cell counterparts (12). In the present study, soluble NADH₂ oxidases were purified from extracts of spores and vegetative cells so that their properties might be compared.

Materials and Methods³

Organism and Cultural Conditions

B. cereus T QMB 1590 was furnished by H. S. Levinson, Pioneering Research Division, U.S. Army Natick Laboratories, Natick, Massachusetts. Spores and vegetative cells were produced in 30-liter quantities of G medium as described by Hashimoto *et al.* (10). Vegetative cells were harvested after 5 hours incubation, washed once with 0.05 M tris(hydroxymethyl)aminomethane (Tris) chloride buffer, pH 7.8, and stored overnight in liquid nitrogen. Spores were harvested by continuous

centrifugation at 4 °C and cleaned using the liquid two-phase system (Y) described by Sacks and Alderton (16). Repeated washings with cold demineralized water resulted in complete removal of vegetative cells and their debris as judged by phase optics. Spores were then freeze-dried and stored at room temperature.

Preparation of Soluble Extracts

Spores and vegetative cells were disrupted by one of two methods. A mixture containing 2 g of spores or vegetative cells and 30 g of No. 380-5005 Superbrite glass beads (Minnesota Mining and Manufacturing Co., St. Paul, Minn.) in 20 ml of 0.05 M Tris chloride buffer, pH 7.8, was subjected to sonication (Branson Sonifier Model S-75, Branson Instruments, Inc., Danbury, Conn.) in a stainless steel cup with ice-water bath cooling. A total of 7.5 minutes sonic oscillation, consisting of five 1.5-minute pulses at 8 amperes with 2-minute cooling intervals, was sufficient to disrupt 95% of the spores. Vegetative cells were given an identical treatment.

Alternatively, spores and vegetative cells were ruptured with a French pressure cell (American Instruments Co., Silver Spring, Md.) operating between 16 000 and 18 000 pounds per square inch (p.s.i.). Spores were broken by a single treatment with the French pressure cell if first sensitized and exposed to lysozyme (Sigma Chemical Co., St. Louis, Mo.) as described by Deutscher and Kornberg (4).

Glass beads and cell debris were recovered by centrifugation at 30 000 × g. The supernatant was further centrifuged for 1 hour at 144 000 × g to remove the remaining particulate matter.

Assay Procedures

Initial rates of NADH₂ oxidation at 25 °C were determined by following the decrease in absorbance at 340 mμ using a Beckman DU-2 spectrophotometer. The reaction mixture contained 50 μmoles Tris chloride pH 7.8, 0.3 μmole NADH₂, 0.1 μmole FMN, and enzyme in a final volume of 1.5 ml. Enzyme concentrations were chosen to be within the range of linear rate dependence. It was necessary to incubate the enzyme and FMN for 3 minutes at room temperature to observe maximal

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activity. A unit of enzyme activity, defined as the amount of protein causing an absorbance change of 0.001 per minute, corresponds to the oxidation of 0.16 μ moles of NADH₂ per minute. The molar absorbance index of NADH₂ was taken as 6.22×10^3 at 340 m μ (2). Specific activity is the number of units per milligram of protein. Protein was estimated spectrophotometrically (13) or determined by the biuret reaction (7).

Purification of NADH₂ Oxidases

Nucleic acids were removed from soluble extracts with streptomycin sulfate (final concentration 1.5% w/v) and the precipitate between 0.4 and 0.75 ammonium sulfate saturation obtained. This fraction was dialyzed against demineralized water followed by adsorption onto hydroxylapatite (Bio Gel HTP, Calbiochem, Los Angeles, Calif.) in centrifuge tubes. Elution was accomplished with stepwise increases in the concentration of phosphate buffer, pH 7.0, each followed by centrifugation at 12 000 $\times g$ for 5 minutes. Fractions containing NADH₂ oxidase activity were combined and concentrated by membrane ultrafiltration. Preparative acrylamide gel disc electrophoresis (Canal Industrial Corp., Bethesda, Md.) as outlined by Ornstein (15) and Davis (3) and modified by Jovin *et al.* (11), was used for the final purification of soluble NADH₂ oxidase. Stacking and separating gels contained 3.5 and 12% acrylamide, respectively. The column buffer (pH 8.3) contained 3.0 g Tris and 14.4 g glycine per liter and the elution buffer was 0.03 M Tris chloride, pH 7.8. Ammonium persulfate was used exclusively as the polymerization catalyst.

Results

Enzyme Purification

B. cereus T spore and vegetative cell NADH₂ oxidases were purified from soluble extracts

in a three-step procedure consisting of ammonium sulfate fractionation, ion exchange on hydroxylapatite, and acrylamide gel disc electrophoresis. Results of the purification procedures are outlined in Table I for spores and vegetative cells. Most of the activity was obtained in the fraction precipitating between 40 and 75% ammonium sulfate saturation. This fraction was further purified when adsorbed on hydroxylapatite and the eluate between 0.01 and 0.05 M phosphate buffer, pH 7.0, collected. Acrylamide gel disc electrophoresis of hydroxylapatite fraction resulted in the final purification of the soluble NADH₂ oxidases. As indicated, this treatment separated the NADH₂ oxidase activity of spore extracts into two fractions while that of vegetative cell extracts was found in a single peak.

The acrylamide gel electrophoresis elution patterns are presented in Fig. 1 for spore and Fig. 2 for vegetative cell extracts. Enzyme in the first spore peak was purified 250-fold over the initial soluble extract while that in the second peak was purified 375-fold. Analytical acrylamide gel electrophoresis (pH 8.3) indicated that the first spore peak consisted of a single component while the second contained two major and two minor protein bands. Of the total activity recovered, about 30% consistently appeared in peak one while 70% was in peak two.

TABLE I
Typical purification of soluble NADH₂ oxidase from spores and vegetative cells of *Bacillus cereus* T

Procedure	Protein, mg	Specific activity	Total units	Purification factor
From spores				
Soluble extract	280	100	28 000	—
Nucleic acid removed	230	120	27 500	1.2
Precipitate, 40–75% (NH ₄) ₂ SO ₄ saturation	30	650	19 500	6.5
Hydroxylapatite eluate	11	1 500	16 500	15.0
Electrophoresis				
Peak one max.	0.1	25 000	2 500	250.0
Peak two max.	0.1	37 500	3 800	375.0
From vegetative cells				
Soluble extract	390	150	58 000	—
Nucleic acid removed	350	160	56 000	1.1
Precipitate, 40–75% (NH ₄) ₂ SO ₄ saturation	90	480	43 000	3.2
Hydroxylapatite eluate	32	1 200	38 000	8.0
Electrophoresis, max.	0.2	12 000	2 400	80.0

Enzyme found in the single peak resulting from acrylamide gel electrophoresis of preparations from vegetative cells was purified 80-fold with respect to the initial soluble extract. Analytical acrylamide gel electrophoresis revealed one major and two minor protein bands in this fraction. The preparative acrylamide gel electrophoresis patterns shown in Figs. 1 and 2 are typical for extracts prepared from spores and vegetative cells whether disrupted by sonication or by use of the French pressure cell.

Identification of the Two Spore Enzymes

Soluble extracts from spores and vegetative cells, each purified through the hydroxylapatite step and each containing 30 mg protein, were mixed and subjected to acrylamide gel electrophoresis. Two peaks of activity resulted, with 55% of the total residing in the first peak while 45% appeared in the second. Recovered activity

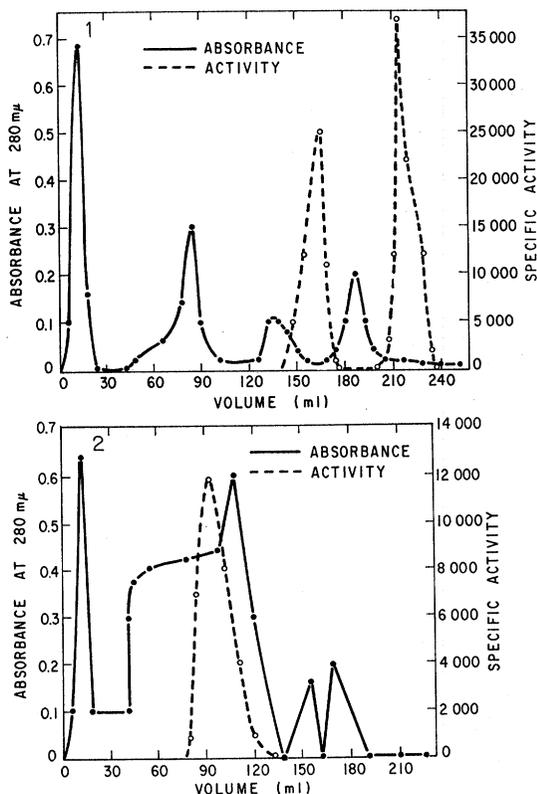
in peak one was increased by an amount equal to the recovery expected had the vegetative cell extract been electrophoresed alone. The presence of vegetative cell extract resulted in no increase in the activity recovered in peak two. These results indicate that enzyme in the first spore peak is identical in electrophoretic mobility on acrylamide gel with vegetative cell soluble NADH₂ oxidase while the second peak of activity found in spore extracts is unique to the spore.

Discussion

Several investigators have presented evidence that spore enzymes possess heat resistance and kinetic, immunologic, and physical properties which distinguish them from their vegetative cell counterparts (8, 9, 14, 17). Others have questioned the validity of these claims on the basis of the impurity of preparations and the variability of enzyme properties, and conclude that enzymatic activities in vegetative cells and spores probably reside in identical proteins (12).

In this study, acrylamide gel electrophoresis of soluble extracts prepared from mature spores of *B. cereus* T revealed the presence of two distinct components with NADH₂ oxidase activity. Soluble extracts prepared in an identical manner from 5-hour cultures of vegetative cells contained only one of the components found in spore extracts. The minor spore component was found to be identical with the vegetative cell enzyme in electrophoretic mobility on acrylamide gel.

The detection of vegetative cell enzyme in spore extracts is not surprising since entrapping of vegetative cell constituents occurs when the forespore is pinched off from the mother cell (9). Extensive washings of spore suspensions used in the present study removed all vegetative cells and their debris as confirmed by microscopic examination, precluding the possibility that spore peak one arose from contaminating vegetative cells. However, the finding in spore extracts of a second electrophoretically distinct soluble NADH₂ oxidase which constitutes 70% of recovered activity suggests either *de novo* synthesis of a characteristic spore enzyme or conversion of existing vegetative cell enzyme during sporulation as reported by Sadoff and Celikkol for fructose-1,6-diphosphate aldolase of *B. cereus* (18).



FIGS. 1 and 2. Elution pattern showing absorbance at 280 mμ (solid line) and specific activity (broken line) of spore (Fig. 1) and vegetative cell (Fig. 2) extract eluted during preparative acrylamide gel electrophoresis.

It is considered unlikely that the second spore enzyme arises as a result of treatments, such as prolonged sonic oscillation, used during disruption procedures. Identical sonication treatments of vegetative cells failed to generate the active fraction unique to spore extracts. In addition, extracts obtained from spores opened by sensitization and exposure to lysozyme contained both NADH₂ oxidase components characteristic of extracts prepared from spore sonicates.

Ashton and Blankenship (1) have investigated some of the physical and chemical properties of *B. cereus* T spore and vegetative cell soluble NADH₂ oxidase. The results of this study further indicate that the major spore component differs significantly from its vegetative cell counterpart.

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